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Role of the AtRad1p endonuclease in homologous recombination in plants

Sandra Dubest, Maria E. Gallego & Charles I. White+

UMR 6547 BIOMOVE, Université Blaise Pascal, 24 ave. des Landais, 63177 Aubière, France

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Using a specific recombination assay, we show in the plant *Arabidopsis thaliana* that AtRad1 protein plays a role in the removal of non-homologous tails in homologous recombination. Recombination in the presence of non-homologous overhangs is reduced 11-fold in the *atrad1* mutant compared with the wild-type plants. AtRad1p is the *A. thaliana* homologue of the human Xpf and *Saccharomyces cerevisiae* Rad1 proteins. Rad1p is a subunit of the Rad1p/Rad10p structure-specific endonuclease that acts in nucleotide excision repair and inter-strand crosslink repair. This endonuclease also plays a role in mitotic recombination to remove non-homologous, 3′-ended overhangs from recombination intermediates. The *Arabidopsis atrad1* mutant (*uvh1*), unlike *rad1* mutants known from other eukaryotes, is hypersensitive to ionizing radiation. This last observation may indicate a more important role for the Rad1/Rad10 endonuclease in recombination in plants. This is the first direct demonstration of the involvement of AtRad1p in homologous recombination in plants.

INTRODUCTION

Plants need sunlight for photosynthesis; however, they are thus exposed to the harmful effects of solar ultraviolet (UV) radiation. The two major DNA lesions produced by UV irradiation are cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6,4) pyrimidinone dimers (reviewed by Mitchell and Nairn, 1989; Friedberg *et al.*, 1995). If the lesions are not removed, they both block DNA replication and transcription (Protic-Sabljic and Kraemer, 1985) and also cause mutations.

Nucleotide excision repair (NER) is responsible for processing DNA lesions that generate major distortion in the helical DNA structure, such as these UV photoproducts and bulky covalent chemical adducts (reviewed by Friedberg *et al.*, 1995). The biochemical steps constituting this repair pathway have been characterized in *Escherichia coli*, yeast and mammalian systems (Aboussekhra and Wood, 1994; Laat *et al.*, 1999), but much less information is available for plants (for reviews, see Britt, 1999; Tuteja *et al.*, 2001). Following specific recognition of the damage and formation of a pre-incision complex, a single-strand oligonucleotide containing the damage site is excised and the resulting gap filled in and sealed (Friedberg *et al.*, 1995). In *Saccharomyces cerevisiae*, two structure-specific endonucleases nick the damaged DNA strand, Rad1p/Rad10p 5′ and Rad2p 3′ of the damaged site (Davies *et al.*, 1995). The Rad1p/Rad10p endonuclease consists of a heterodimer of Rad1p and Rad10p (Bailly *et al.*, 1992; Bardwell *et al.*, 1992, 1993) and has been shown to incise DNA specifically at 5′-double-strand/3′-single-strand junctions (Tomkinson *et al.*, 1993; Bardwell *et al.*, 1994).

Rad1 and Rad10 proteins exhibit significant amino acid conservation with the mammalian Xpf and Ercc1 proteins, respectively (Brookman *et al.*, 1996; Sijbers *et al.*, 1996). The gene *RAD1* also has homologues in *Schizosaccharomyces pombe* (*RAD16*; Carr *et al.*, 1994) and *Drosophila melanogaster* (*MEI-9*; Sekelsky *et al.*, 1995). *AtRAD1*, a homologue of *RAD1*, has recently been described as a single-copy gene in the plant *Arabidopsis thaliana* (Fidantsef *et al.*, 2000; Gallego *et al.*, 2000; Liu *et al.*, 2000) and shown to be important for the removal of 6-4 photoproducts from UV-irradiated plants (Liu *et al.*, 2000). These authors have also shown that the *Arabidopsis uvh1* mutant carries a single base pair substitution in the *atrad1* gene, leading to mis-splicing of the *atrad1* mRNA in mutant plants (Liu *et al.*, 2000). *In vitro* assays have confirmed this role in NER and also indicated a possible role for AtRad1 protein (AtRad1p) in the repair of oxidative DNA damage (Li *et al.*, 2002). A single *RAD10* homologue is found in the fully sequenced *Arabidopsis* genome (locus At3g05210), and a *RAD10* homologue has also been described in lily (Xu *et al.*, 1998).

The Ercc1/Xpf endonuclease plays an essential role in NER. Ercc1$^{-/-}$ mice show a more severe phenotype than other NER-deficient mouse models, apparently associated with a premature replicative senescence in the Ercc1$^{-/-}$ cells (Weeda et al.,

+Corresponding author. Tel: +33 4 73 40 79 78; Fax: +33 4 73 40 77 77; E-mail: chwhite@univ-bpclermont.fr

1997). Moreover, Xpf-deficient mammalian cells and AtRad1p-deficient plants, unlike other NER-deficient cells, are sensitive to crosslinking agents (Westerveld *et al.*, 1984; Hoy *et al.*, 1985; Gallego *et al.*, 2000). Unlike *xpf* and yeast *rad1* and *rad10* mutants, the *atrad1* mutant (*uvh1*) is also hypersensitive to ionizing radiation (Harlow *et al.*, 1994; Jenkins *et al.*, 1995; Jiang *et al.*, 1997; reviewed by Paques and Haber, 1997). These observations underline the importance of a potential role for AtRad1p in recombination, as well as its role in NER. A number of reports have shown Rad1p and Rad10p to have a role in mitotic recombination (reviewed by Paques and Haber, 1997). Mutations in *RAD1* and *RAD10* reduce intra-chromosomal recombination between directly repeated sequences 8-fold and also decrease the efficiency of homologous integration of linear DNA fragments and circular plasmids (Klein, 1988; Schiestl and Prakash, 1988, 1990; Prado and Aguilera, 1995). The *rad1* and *rad10* mutations are epistatic to one another (Schiestl and Prakash, 1988, 1990; Saffran *et al.*, 1994), so the recombination role depends upon the heterodimer formation and most likely on its endonuclease function. Yeast excision repair mutants other than *rad1* and *rad10* do not show these recombinational phenotypes, further demonstrating the specific role of Rad1p/Rad10p in recombination (Ivanov and Haber, 1995).

Intra-chromosomal recombination between tandem, direct repeats occurs most efficiently via single-strand annealing (SSA) following the formation of a DNA double-strand break (DSB) between, or within, the repeated sequences (discussed by Paques and Haber, 1997). Exonucleolytic resection of the 5′-ended strands at the DSB produces two complementary, 3′-ended single strands. These strands anneal and, after excision of any non-homologous 3′-ended overhangs and new DNA synthesis, ligation restores two continuous strands. Rad1p and Rad10p are required for the removal of these 3′ non-homologous tails and for the removal of non-homologous sequences during DSB-induced recombination between plasmid inverted repeats (Fishman-Lobell and Haber, 1992; Ivanov and Haber, 1995; Prado and Aguilera, 1995; Saparbaev *et al.*, 1996; Paques and Haber, 1997; Sugawara *et al.*, 1997; Colaiacovo *et al.*, 1999). Similarly, Ercc1p is required to remove non-homologous tails from ends-in gene targeting constructs in CHO cells (Adair *et al.*, 2000; Sargent *et al.*, 2000) and for targeted gene replacement in mouse embryonic stem cells (Niedernhofer *et al.*, 2001). Interestingly, Niedernhofer *et al.* (2001) suggest that the role of Rad1p/Rad10p in nicking synapsed recombination intermediates leads to the formation of a DSB in the recipient chromosomal DNA, thus permitting insertion of non-homologous DNA into the chromosome.

As part of our analysis of DNA recombination mechanisms in plants, we present here results of a specific assay showing that AtRad1p plays a role in permitting productive recombination between DNA molecules in *A. thaliana* in the presence of non-homologous tails.

RESULTS AND DISCUSSION

Over the last decade, a number of studies have contributed to our understanding of the apparent conservation of recombination-associated proteins and recombination mechanisms between plants and other eukaryotes (reviewed in Britt, 1999; Gorbunova and Levy, 1999; Mengiste and Paszkowski, 1999; Vergunst and Hooykaas, 1999; Tuteja *et al.*, 2001). However, the lack of identified mutant plants for recombination functions has meant that experimental verification of the roles of specific proteins in recombination in plants is lagging behind the identification, based on analogy, of the corresponding genes. In the present study, we tested specifically the involvement of the AtRad1p endonuclease in removing non-homologous overhangs to permit productive homologous recombination between linear DNA molecules in *A. thaliana*. Our assay involves observing transient expression of recombined plasmids following biolistic transformation of *Arabidopsis* leaves.

AtRad1p is not needed for homologous recombination in the absence of non-homologous overhangs

In this 'no-tails' experiment, we addressed the question of whether AtRad1p is required for recombination between overlapping homologous DNA regions in the absence of non-homologous overhangs (as indicated in Figure 1).

In order to monitor inter-molecular recombination, plasmids pcw344 and pcw345 were linearized with *Eco*RI and *Bsm*I, respectively, and used to co-transform leaves from wild-type and *atrad1* plants (as described in detail in Methods and shown in Figure 1). Plasmid pcw344 contains only the region encoding the C-terminal region of the β-glucuronidase (GUS) gene and the nopaline synthetase (NOS) terminator, whereas pcw345 contains the 35S promoter and the region encoding the N-terminal region of GUS. The overlapping homology region is

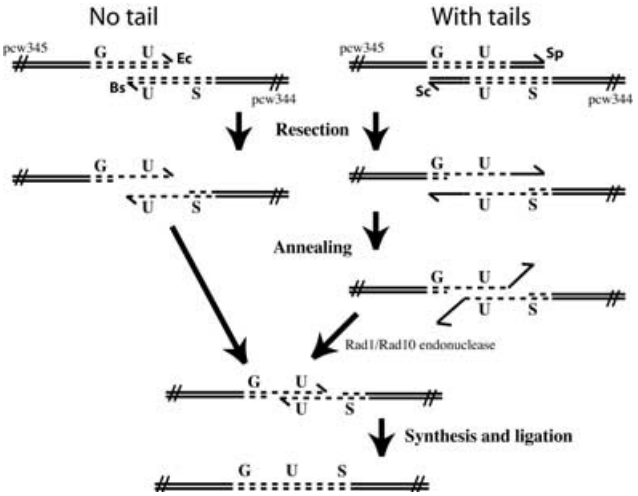

Fig. 1. Recombination assay. Linear recombination substrates were prepared from pcw344 and pcw345 plasmids by restriction with enzymes that leave ('with tails') or do not leave ('no tails') non-homologous overhangs. The 'no tails' test was carried out with pcw345/*Eco*RI and pcw344/*Bsm*I and the 'with tails' test with pcw345/*Sap*I and pcw344/*Sca*I. The relevant linearized plasmids were used to co-transform leaves from either wild-type or *atrad1* plants and the number of recombinant spots per leaf counted. Resection of the linear molecules permits annealing of complementary homologies. The annealed 3′-ended strands of 'with tails' plasmids have unpaired, non-homologous overhangs. The Rad1/Rad10 endonuclease removes these, permitting re-synthesis and ligation to generate the recombined product. Both DNA strands are shown with arrowheads indicating the 3′ ends. Ec, *Eco*RI; Bs, *Bsm*I; Sp, *Sap*I; Sc, *Sca*I.

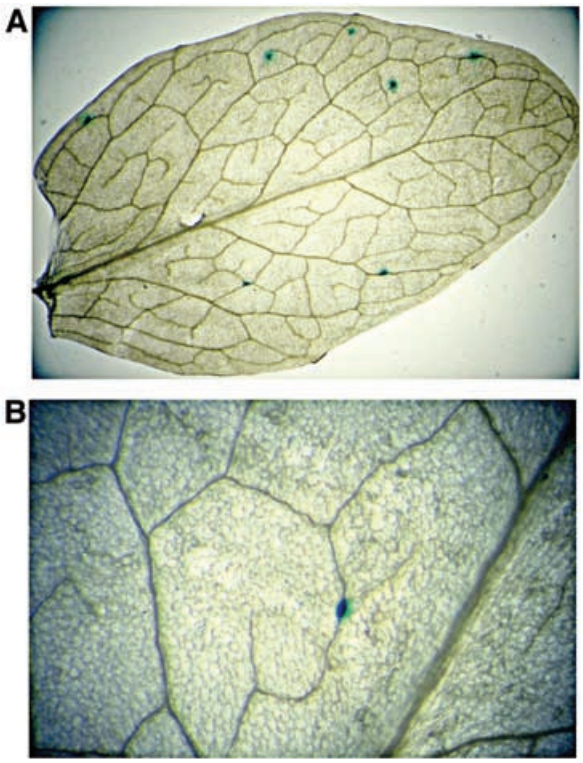

Fig. 2. Examples of blue spots after GUS staining: (**A**) *atrad1* leaf transformed by pGUS23 and (**B**) an example of a recombinant blue spot arising from recombination (*atrad1* 'no tails').

590 bp long, which has previously been shown to be sufficient for efficient recombination in analogous assays (Puchta and Hohn, 1991). GUS activity was subsequently detected as blue spots following histochemical treatment. Examples of transformed leaves showing blue GUS spots are shown in Figure 2.

As shown in Tables I and II and Figure 3, the frequencies of recombination events do not differ significantly between the two lines. Thus AtRad1p is not required for DSB repair by homologous recombination in the absence of non-homologous overhangs ('no tails').

No recombinant spots were detected in control transformations carried out in parallel with the same quantities of the two plasmids individually (data not shown). Transformation efficiency was controlled in parallel by transforming with pGUS23, which contains the entire GUS transcription cassette. In order to produce a quantifiable number of spots, 10-fold less plasmid DNA was used (5 µg versus 50 µg in the recombination assays). The numbers of GUS$^+$ spots obtained with pGUS23 transformation of *atrad1* and wild-type leaves were similar; thus, these two plant lines were transformed with similar efficiency (Tables I and II).

AtRad1p is required for homologous recombination in the presence of non-homologous overhangs

In *S. cerevisiae*, the Rad1p/Rad10p endonuclease is required for the removal of 3'-ended, non-homologous single-stranded tails from SSA intermediates during DSB-induced recombination between direct repeats (Fishman-Lobell and Haber, 1992; Ivanov and Haber, 1995; Prado and Aguilera, 1995; Sugawara *et al.*, 1997).

To test the role of AtRad1p in the elimination of non-homologous tails during SSA, a second recombination test was carried out. *atrad1* and wild-type plants were co-transformed with pcw344/*Sca*I and pcw345/*Sap*I (as described in Methods and shown in Figure 1). The overlapping homology region is 590 bp in both cases, to which pcw344/*Sca*I adds a non-homologous tail of 816 bp and pcw345/*Sap*I a tail of 233 bp.

The *atrad1* mutant plants show significantly (11-fold) less recombinant spots than the wild-type plants ('with tails', Tables I and II and Figure 3). Thus, AtRad1p is implicated in DSB repair by homologous recombination in the presence of non-homologous tails. We note that, although the absence of AtRad1p strongly reduces recombination in the presence of non-homologous tails, it does not eliminate it altogether in our assay. Thus, a minor, AtRad1p-independent pathway for these events must exist. Another possible factor contributing to these AtRad1-independent events might be due to exonucleolytic action on the linear DNA substrates. This possibility is supported by our observation that, in the wild-type leaves, the 'with tails' constructs give more

Table I. AtRad1p removes overhanging tails for homologous recombination: numbers of GUS$^+$ blue spots per leaf

pGUS23		No tails		With tails	
wild type	*atrad1*	wild type	*atrad1*	wild type	*atrad1*
17	35	7	8	11	1
24	28	3	7	13	2
28	23	11	4	10	2
57	41	6	3	16	0
30	23	12	9	18	1
30	31	6	5	14	0
36	45	7	6	16	5
31	21	4	4	15	0
44	43	5	4	18	0
21	31	5	5	20	2
23		2	1	24	2
		2	2	12	2
		1	3	16	0
		11	4	14	2
		3	3	10	1
		3	2	13	1
		5	1	16	2
			6	11	1
			2	8	2
			4	6	0
				14	

Wild-type and *atrad1* mutant leaves were transformed with pGUS23 (transformation efficiency control) or with 'with tails' or 'no tails' recombination substrates. After histochemical staining, blue spots were counted under a dissecting microscope. Counts are from at least three different transformations.

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Table II. AtRad1p removes overhanging tails for homologous recombination: χ^2 (1 degree of freedom) values for the null hypothesis that all leaves from a given line are from the same population with respect to number of blue spots

	Wild type			*atrad1*				Chi squared	
DNA	*n*	*N*	*X*	*n*	*N*	*X*	Reduction	wild type	*atrad1*
pGUS23	11	341	31.0	10	321	32.0	0.97	0.82	0.40
No tails	17	93	5.5	20	83	4.2	1.3	0.53	0.80
With tails	21	295	14.1	20	26	1.3	10.85	17.19	20.00

Non-parametric statistical analysis was carried out as described in Methods. In the 'with tails' transformation, numbers of recombinants (blue spots) in the *atrad1* mutant are significantly (10.85-fold) reduced, permitting the rejection of the null hypothesis. No significant differences are observed in the transformation efficiency (pGUS23) or the 'no tails' control transformations.
n, number of leaves analysed; *N*, total number of blue spots; *X*, mean number of recombination events per leaf.

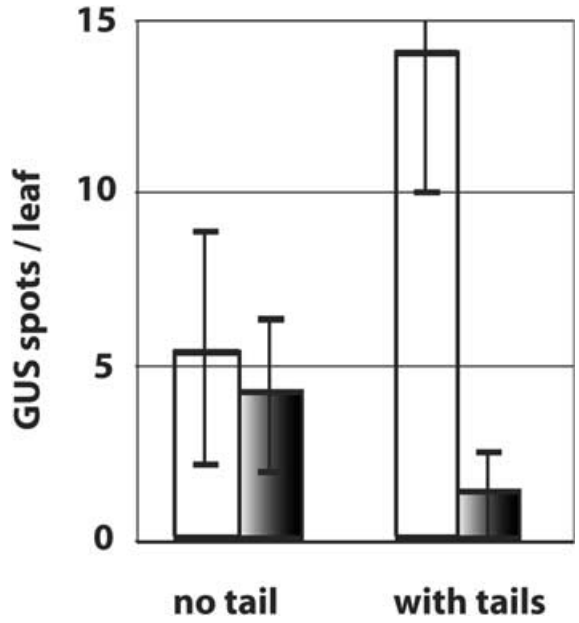

Fig. 3. Recombination in *atrad1* and wild-type leaves. Effect of the non-homologous tails on the recombination rate in wild-type (white bars) and *atrad1* mutant (grey bars) plants. The presence of non-homologous overhangs ('with tails') specifically reduces the frequency of recombinant spots in the *atrad1* mutant. No significant difference between mutant and wild-type plants is seen in the absence of the non-homologous overhangs ('no tails'). Error bars are ± 1 standard deviation.

recombinant spots than the 'no tails' constructs. Thus, the tails may act as a 'buffer' protecting the homologous GUS sequences from exonuclease degradation prior to recombination. That such an exonuclease activity does not affect our conclusions concerning the role of AtRad1p in our experiments is demonstrated by the equivalent recombination efficiencies in the wild-type and mutant leaves with the 'no tails' substrates (Figure 3).

In conclusion, we show here that the *Arabidopsis* Rad1p homologue acts on recombining DNA molecules to permit productive homologous recombination in the presence of non-homologous DNA overhangs. We are now testing the implication of AtRad1p in other forms of homologous recombination in *Arabidopsis*, such as gene targeting and spontaneous and induced intra-chromosomal recombination.

METHODS

Strains and growth conditions. *Arabidopsis thaliana* were ecotype Columbia (Col). The *atrad1* UV-hypersensitive mutant (uvh1-1; Harlow *et al.*, 1994) was obtained from the Nottingham Arabidopsis Stock Center (CS3819; NASC: online catalogue available at http://nasc/nott.ac.uk/home.html). After sterilization with sodium hypochlorite (7%, w/v), *atrad1* and wild-type seeds (ecotype col4) were sown on germination medium [Murashige and Skoog basal medium with vitamins (Sigma), supplemented with 30 g/l sucrose, pH 5.8]. Plates were incubated in a growth chamber at 22°C (16 h light, 8 h dark). *atrad1* mutants were verified both by Southern hybridization and UV hypersensitivity (data not shown).

Plasmid constructs. As the control for transformation efficiency, we used plasmid pGUS23 (Puchta and Hohn, 1991), which contains the cauliflower mosaic virus (CaMV) 35S promoter, the GUS gene and the NOS transcription terminator.

The plasmid pcw344 was constructed by digesting pGUS23 with *Nde*I and *Sna*BI to eliminate the 35S promoter and the GUS N-terminal region. The resulting plasmid was blunt-ended with T4 DNA polymerase, purified through low-melting-point agarose and re-circularized with T4 DNA ligase.

The plasmid pcw345 was obtained by *Sfu*I–*Eco*RI digestion of plasmid pGUS23C1 (a deletion derivative of pGUS23; Puchta and Hohn, 1991), which contains the 35S promoter, the GUS N-terminal region and the NOS terminator. This eliminated the NOS terminator. The resulting plasmid was purified, blunt-ended and circularized as above

Biolistic transformation and recombination assay. Leaves from 3- to 4-week-old wild-type and *atrad1* mutant plants were placed adaxial side up on solid germination medium in Petri dishes. Gold beads (Sigma; 0.6 µm diameter) were washed in water then rinsed and resuspended in ethanol at 0.1 µg/µl. To 50 µl of beads, 50 µl (50 µg) of plasmid DNA, 250 µl of 2.5 M $CaCl_2$ and 100 µl of 0.1 M spermidine (Sigma) were added. After 5 min on ice, the beads were pelleted with a brief centrifugation and 400 µl of supernatant removed.

For each transformation, 5 µl (10%) of the resuspended, DNA-coated gold beads were used with a helium particle gun (6 bars, 28 kPa, 13 cm from the leaves; Finer *et al.*, 1992). Leaves were then placed in a growth chamber at 22°C (16 h light, 8 h dark) for 1 or 2 days and the GUS activity assay carried out as described by Jefferson *et al.* (1987). The number of blue spots

per leaf was determined by visual observation using a dissecting microscope.

Transformation efficiency was controlled by transforming wild-type and mutant plants with 5 µl (5 µg) of pGUS23. The lower quantity of plasmid DNA (5 µg versus 50 µg in the recombination assays) was used to reduce the number of spots per leaf in order to facilitate counting in the controls.

Plasmids pcw344 and pcw345 were linearized with *Bsm*I or *Eco*RI, respectively, to prepare the 'no-tail' recombination substrate. 'With tail' substrates were prepared by digesting pcw344 and pcw345 with *Sca*I or *Sap*I, respectively. In all cases, the overlapping homology region is 590 bp, to which pcw344/*Sca*I adds a non-homologous tail of 816 bp and pcw345/*Sap*I a tail of 233 bp. In both 'with-tails' and 'no-tails' experiments, the transformation was carried out with 25 µl (25 µg) of each plasmid (pcw344 and pcw345).

Statistical analysis. The data on the number of recombination events per transformed leaf were analysed non-parametrically (with the kind advice of Dr E.J. Louis, University of Leicester, UK). All leaves analysed from a given line were ranked by the number of blue spots for each set of leaves analysed. On the null hypothesis that the plants analysed from each line belong to the same distribution, half should be below the mid-point of the ranking and half above. χ^2 (1 degree of freedom) values were calculated for the wild-type and *atrad1* mutants from each type of transformation (Table II).

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